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Introduction

This contract was designed to investigate the basic causal mechanisms responsible for the loss of vascular tone during alterations in transmural distending pressure consistent with that accompanying shifts to a no-gravity environment. The ionic and cellular feedback relationships operating to effect the vascular decompensatory modifications were examined in an effort to reveal procedures that could be implemented as protective measures guarding against vascular collapse when returning from a weightless environment to that of the earth's gravity.

Since the hydrodynamic stimulus for normal vascular tone is transmural distending pressure, appropriate challanges to a skeletal muscle vascular bed were effected by graded reductions in perfusion pressure. The microvascular dimensions and responsivity were monitered during such changes and subsequently the cellular constituents responsible for the vascular adjustments were analyzed utilizing the electron microprobe located in the Manned Spacecraft Center Clinical Laboratories.

Method of Approach

Surgical Procedures: The microcirculatory preparation used was an extensive modification of the Baez preparation of the rat cremaster, a thin skeletal muscle (approximately 250 microns thick in a 100 gm rat) with two layers of muscle fibers oriented obliquely to each other.

The rats were anesthetized intraperitoneally, with a warm solution of 10% urethane and 2% chloralose in physiological saline. This solution has been demonstrated by us to provide for the best maintenance of vascular tone and vasomotion, in contrast to other anesthetics.

The animals were placed upon a special mounting board and a tracheostomy performed with a tube which had incorporated into its wall a heated thermistor, serving as a pneumotachograph when connected to the appropriate electronic circuitry. Rectal temperature was monitored with a thermistor probe and maintained at 38°C by an electrically heated mat.

An incision was made along the ventral suface of the scrotum and the cremaster muscle and intact testis were microdissected of surrounding connective tissue. The cremaster was incised along its ventral aspect with care

being taken not to damage the underlying testis. The testis was then freed from the cremaster and extirpated. All surgery was performed utilizing a micro heat cautery and a stereo dissecting microscope. The cremaster was spread over an electrically heated pedestal, 1.8 cm diameter, and maintained between 34 and 35°C. The in situ temperature of the cremaster had previously been determined by passing a thermistor through the peritoneum, down into the scrotum between the testis and cremaster.

A round 1.8 cm #2 microslide cover slip was positioned atop the cremaster. In this manner, the cremaster was not subjected to equilibration with environmental gases or ionic disturbances resulting from continual washing with exogenous bathing solutions.

After the preparatory surgery, a waiting period of approximately one hour allowed the cremaster arterioles to regain tone and vasomotion.

The cremaster vasculature was observed at powers from 50 to 600X with a microscope modified to accept long working distance optics and a high intensity, fiber optic illuminator containing no infrared and only minimal

near infrared radiation. The phototube of the microscope projected to the front surface of a vidicon tube in a lenseless TV camera. The electronic video image was recorded on a one-half inch helical scan video recorder and viewed on a 23 inch television monitor.

The carotid artery was cannulated for a direct recording of systemic arterial pressure and heart rate. It had previously been determined in this laboratory that, after initial transient, cannulation of one carotid artery does not produce an alteration in systemic hemodynamic parameters.

Fixation Procedures: The intial method of fixing the rat cremaster muscle in vivo was by perfusing or suffusing with Karnovsky's FG fixative.

The methods considered were: 1) unidirectional perfusion from beneath the cremaster muscle preparation; 2) bidirectional perfusion from the top and bottom of the preparation; and 3) intra-arterial (i.a.) injection of the fixative through the femoral artery.

In the unidirectional perfusion technique the cremaster was covered with a microscope slide cover slip

(1.8 cm - diameter). The fixative was pumped up through holes (0.4 mm in diameter) in the top of the pedestal. Holes of this size are occluded easily by extravasated erythrocytes. It was found in the initial studies that some areas of the cremaster began to fix before others, due to insufficient numbers of holes. The exhisting holes were enlarged to 0.6 mm diameter and others of the same size were drilled in the pedestal top such that no part of the cremaster was more than 1 mm distant from a hole.

Using the cessation of blood flow in the arterioles under observation as a rough index of tissue fixation, the fixation time for the unidirectional perfusion varied from 2 to 6 minutes. These time intervals for fixation were a function of the geometrical relationships between the tissue and pedestal top and the hydraulics of the perfusion system. Air pockets forming at the top of the pedestal reservoir prevented areas above the air pockets from being fixed at the same rate as areas not overlying air pockets. More air escape holes were drilled in the outer portion of the pedestal. This alleviated the problem to some degree but air pockets were still observed occasionally.

When holes clogged with erythrocytes were observed they were usually surrounded by other occluded holes.

Here again vessels overlying the group of clogged holes would present longer fixation times. In future experiments this problem should be corrected by covering the pedestal with Saran Wrap until the muscle is mounted atop the pedestal and by employing repetitive irrigation techniques. It is possible that the pedestal top could be covered with a membrane permeable for the fixation but impermeable to erythrocytes.

In early trials it was observed, utilizing the unidirectional perfusion method, that as increasing amounts of fixative were perfused, the cremaster was distended upward, thus possibly upsetting any homeostatic equilibrium that had been obtained. This problem was solved in later experiments by decreasing the perfusion rate (to .07 ml/mm) resulting in no apparent shift in tissue position.

In an attempt to shorten the fixation time it was decided to employ a preparation with no cover slip and to perfuse from both sides of the cremaster. Fixative was forced up from the reservoir thru the pedestal holes and at the same time suffused onto the cremaster via irrigation tubes. Fixation times using this technique ranged from 1 to 2 minutes, an improvement, time-wise, over the

unidirectional perfusion. Air pockets, holes clogged with erythrocytes and distention of the cremaster were also less of a problem using this technique. The overlying layer of fixative presented some small problems in focusing, and in general decreased the resolution, but not below tolerable limits.

A third technique - intra-arterial injection of the fixative upstream into the femoral artery - was then attempted. In these experiments blood flow usually stopped prior to a one minute mark after a 15 second i.a. infusion of fixative at approximately 0.07 ml/min. It is believed that this method fixes only the blood vessel walls and contents. Cremaster muscles fixed by this procedure were generally more flexible in relation to cremasters fixed by the two preceding methods, thus indicating insufficient fixation of the skeletal muscle by this method. Since the vessel walls are fixed internally preserving their ionic environment, the skeletal muscles surrounding the blood vessels may be post-fixed to allow proper sectioning for the microprobe. It is unlikely that the pressure in the cremaster arteries and arterioles was increased by this method, in view of the small perfusion rate.

Cremasters fixed via the above described techniques were flown to Houston and analyzed with the microprobe. Contrary to prior consultative advice and reports in the literature, it was found that Karnovsky's FG fixative did not maintain the *in vivo* ionic concentrations but rather rendered the cell membrane extremely porous, it particular, with respect to potassium. It seemed likely that any fixation procedure based upon chemical binding would produce the same result. For this reason we then considered methods of physically preserving the assay tissue.

An asbestos and metal animal support board for the preparation and quick-freezing of the cremaster by liquid nitrogen application was fabricated and evaluated. The support of the experimental animal, including all structures and tissues except the cremaster muscle, was fabricated from asbestos in order that minimal central reflexes and responses would be elicited during the rapid fixation procedure. A hollow stainless steel cylinder (1.8 cm diameter, 2.5 cm height) supported the cremaster and connected via a .6 cm I.D. stainless steel tube to a stainless steel reservoir. The rapid fixation was initiated by pouring liquid nitrogen into the reservoir which in turn immediately filled the cremaster support cylinder. The support cylinder was vented

to atmosphere via a .15 cm I.D. stainless steel tube extending 2 cm above the cremaster level, thus allowing air trapped in the cylinder to escape. This technique allows for an almost instantaneous application of liquid nitrogen to the thin stainless steel in direct contact with the cremaster.

Once the liquid nitrogen was applied and the concomitant fixation of the cremaster tissue observed microscopically, the cremaster was severed from the animal. The cremaster was removed from the support pedestal and immediately placed on dry ice where it remained during shipping to the analysis site at NASA-Houston.

Four samples of cremaster muscle, two controls and two animals under simulated werighlessness (reduced perfusion pressure), were prepared by the above methods. The samples were flown to Houston where they underwent analysis.

The frozen muscles were cut in 8 μ sections, and placed on either glass slides for H & E staining or on silicon discs for microanalysis.

The silicon disc sections were coated with Pd-Au (200 Å) and C (500 Å) and analyzed with the microprobe

in the scanning secondary electron mode. The sections were not protected sufficiently by the coatings used and it was noted that future analyses would be performed with thicker coatings.

Additional frozen sections were left at the Manned Spacecraft Center for future analysis.

Efforts then were turned to perfecting of a method for rapid fixation of the cremaster tissue by microwave irradiation. Late in the period one Litton Model #500 microwave oven was received by the Contractor from NASA-MSC. The laboratory housing the oven has necessarily been re-wired to accomodate the unit with its specialized power connections. No modification of the microwave oven has been necessary.

An animal support board, suitable for use with microwave irradiation has been constructed. This board, which has been tested in several preliminary experiments does not interfere with the irradiation of the tissue, nor does it absorb sufficient energy to render itself a thermal source in the fixation of the tissue.

The cremaster, after being surgically prepared, was

initially fixed in a Litton Model Number 500 microwave oven to preserve the biochemical and ionic balance.

The cremaster muscle was then removed from the animal and placed in Ringer's solution at room temperature. The muscle was cut into halves to facilitate handling and sectioning.

The muscle tissue was placed in an Ames Lab-Tek Model Number D cryostat/microtome at -30°C. The tissue was later embedded in Ames O.C.T. compound on a brass tissue block and placed in the microtome holder for sectioning.

It was determined that sections of 8.0 μ thickness would be the most suitable size for electron microprobe analysis.

Initially, the first few sections cut at 8.0 μ were mounted on a glass slide. Mounting was accomplished by merely touching room temperature slides to the cut tissue sections, where they firmly adhered. These sections were then examined under a light microscope at low power with dark-field illumination to insure that the current sections being cut included portions of the vascular system. When it was determined that the sections on the glass slide contained parts of the vascular system, the next sections

cut from the tissue were placed on polished metal discs cooled to -30°C in the cryostat. These discs with the sections remained in the cryostat to prevent thawing of the tissue sections. Subsequent sectioning of the muscle at different levels and observation at low power under the light microscope continued until 3 good tissue sections were obtained which contained large portions of the vascular network.

The polished discs were then quickly placed in a Mikros (Varian) VE 10 vacuum evaporator before the tissue sections had sufficient time to thaw out and thereby destroy the vascular morphology, especially the vessel walls. The ice crystals were then rapidly sublimed at a pressure of 0.1 to 0.05 u for 30 minutes to insure that all ice was removed. In this manner, liquid water could not form in the cells, which upon exposure to room temperature, would eventually evaporate and cause destruction of the vascular structures.

After the vacuum evaporation, the discs and sections could be exposed to room temperatures without damage to the cellular contents.

The sections were packed in plastic petri dishes with layers of lens tissue paper between the discs and shipped

to NASA for examination with the electron microprobe.

This method of rapid freeze-drying seems to offer the most promising technique for preservation of morphological and biochemical characteristics of the vascular network.

These sections also are awaiting analysis at the Manned Spacecraft Center.

Pressure Measuring System: The Biomedical Engineering Department has designed and constructed several devices for use in measuring pulsatile pressures in microvessels. The pressure measuring system constructed is an extensive modification of that reported by Dr. Curt A. Wiederhelm (Amer. J. Physiol 207:173, 1964). Component devices constructed for this system include:

(1) 1800 Hz Carrier Oscillator
(2) Micropipet Resistance Bridge
(3) FET-Input Carrier Amplifier
(4) Phase Detector
(5) Micropipet Resistance Calibration Tank

In addition, considerable experimentation was undertaken in developing and perfecting an "electromagnetic

pump" capable of supplying the necessary active element for the pipette system.

Recommendations:

We have been informed by the Manned Spacecraft Center that due to the time limitations imposed by the Skylab project, the microprobe will not be available for use on this contract in the near forseeable future. Due to this fact and the difficulties encountered in the preservation of intracellular ionic profiles, it is our recommendation that this project be terminated at the present stage.

Publications:

A copy of a publication resulting from this work is attached to this report. Future publications will give credit to NASA and will likewise be forwarded to the appropriate office.

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Abstracts

Considerations for the Functional Preservation of Vascular Tissue. W. C. ALEXANDER, P. M. HUTCHINS, AND S. L. KIMZEY, NASA, Manned Spacecraft Center, Houston, TX 77058; Bowman Gray School of Medicine, Winston-Salem, NC.

Classical techniques for the fixation of tissue have often assumed accurate preservation of the morphometry and functional dimensions of the microvasculature. Whereas these techniques may serve the purpose for purely morphological studies, it is suggested that these methods do not preserve the functional integrity of the microcirculation. In studies designed to elucidate the relationship between the extra- and intracellular ionic constituency of the vascular smooth muscle cell and its response to hemodynamic alteration, it has been noted that chemical fixatives are inadequate for this purpose. In a series of experiments designed to study such relationships *in vivo* it has been shown that fixation involving chemical stabilization of the cellular structure renders the cell membrane extremely porous, and fails to maintain normal electrolyte distribution. It is known that osmotic changes in the vessel grossly influence the dimensions of the vascular space. Theoretically, the ideal fixative must maintain those features representing the normal anatomical and physiological behaviour of the tissue. For these reasons, our laboratories are currently involved in investigations designed to explore alternative methods of tissue preservation. Although locally applied liquid nitrogen offers a means of accurate and rapid stabilization of tissue structures, this technique is costly, hazardous and extremely inconvenient to apply to specific areas without disruption of the adjacent elements. An alternative approach utilizes focused microwave irradiation to stabilize the cell membrane while maintaining the integrity of vascular tissue.

Comparative Studies on Alpha-Adrenergic Receptors in Rat Blood Vessels. BURTON M. ALTURA, Departments of Anesthesiology and Physiology, Albert Einstein Col. Med., Bronx, N.Y.

Recent work on mouse and cat visceral smooth muscle indicates that there may be differences between the α-adrenergic receptors in the splenic smooth muscle of the two species. To our knowledge no such comparative study has been made on blood vessels. Studies were therefore undertaken with female and male rats using 15 catecholamine analogs and the α-antagonist phentolamine. Rat mesenteric arterioles (MA) were examined *in vivo* using the image-splitter television microscopy system of Baez. Rat aortas (A) and portal veins (PV) were examined *in vitro* under conventional isometric recording conditions. Complete dose–response curves (DRC) for the various catecholamine agonists and analogs were determined on all 3 vessel types in the presence and absence of phentolamine. From these DRC we determined the relative maximum contractile responses (intrinsic activities, i.a.), affinities (ED_{50}) and the apparent dissociation constant (K_B) of the antagonist for the receptor–agonist complexes. Although the i.a. for E and NE = 1.0 on the A and PV, the i.a. for NE on the MA = 0.8. Data indicate that not only do the relative i.a. in a series of 15 catecholamine agonists differ from one type of rat blood vessel to another but that the affinities relative to E and NE also vastly differ. Furthermore, the apparent K_B values of the antagonist for the various receptor–agonist complexes also differ from one vessel type to another. Overall, the data strongly suggest: (1) the existence of different α-receptor types in different blood vessels within a single mammalian species, and (2) that the structure–activity relationships for a series of adrenergic agonists also differ from one vessel type to another.